Adhesion to target cells is disrupted by the killer cell inhibitory receptor

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Killer cell immunoglobulin-like receptors (KIR) inhibit the cytotoxic activity of natural killer (NK) cells by recruitment of the tyrosine phosphatase SHP-1 to immunoreceptor tyrosine-based inhibition motif (ITIM) sequences in the KIR cytoplasmic tail [1]. The precise steps in the NK activation pathway that are inhibited by KIR are yet to be defined. Here, we have studied whether the initial step of adhesion molecule LFA-1-dependent adhesion to target cells was altered by the inhibitory signal. Using stable expression of an HLA-C-specific KIR in the NK cell line YTS [2] and a two-color flow cytometry assay for conjugate formation, we show that adhesion to a target cell expressing cognate HLA-C was disrupted by KIR engagement. Conjugate formation was abruptly interrupted by KIR within less than 5 minutes. Inhibition of adhesion to target cells was mediated by a chimeric KIR molecule carrying catalytically active SHP-1 in place of its cytoplasmic tail. These results suggest that other ITIM-bearing receptors, many of which have no known function, may regulate adhesion in a wide variety of cell types.

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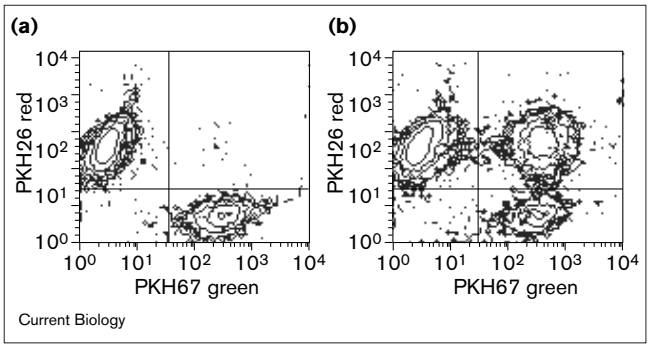
Results and discussion

The NK cell line YTS stably expressing KIR2DL1 [2], an inhibitory KIR specific for the major histocompatibility complex antigen HLA-Cw4, was used to study the effect of ligation of KIR on the formation of conjugates between effector (NK) cells and target cells. As expected, KIR2DL1 in YTS cells (YTS-2DL1) completely blocked cytolysis of the transfected target cell 721.221 expressing HLA-Cw4 (221-Cw4) but not that of the 721.221 transfectant expressing HLA-Cw3 (221-Cw3) (see Supplementary material), an allotype that is not recognized by KIR2DL1 [3,4]. Effector–target cell heteroconjugates were analyzed by two-color flow cytometry (Figure 1). Binding equilibrium was reached by 10 min at 37°C with ~80% of YTS cells bound to target cells (Figure 2a). Within less than 5 min, however, KIR2DL1 disrupted conjugate formation with 221-Cw4 cells such that the number of effector cells in conjugates was reduced to 20–30% (Figure 2a). Conjugate formation of YTS-2DL1 with 221-Cw3 cells was not affected. Similar results were obtained using another pair of target cells expressing HLA-C molecules that are either recognized by KIR2DL1 (HLA-Cw15) or not (HLA-Cw8) (see Supplementary material). Binding of the KIR2DL1-specific immunoglobulin M (IgM) monoclonal antibody HP-3E4 to YTS-2DL1 restored the number of conjugates with 221-Cw4 to a level similar to that obtained with 221-Cw3, implying that the inhibition observed was due to KIR2DL1 (Figure 2b).

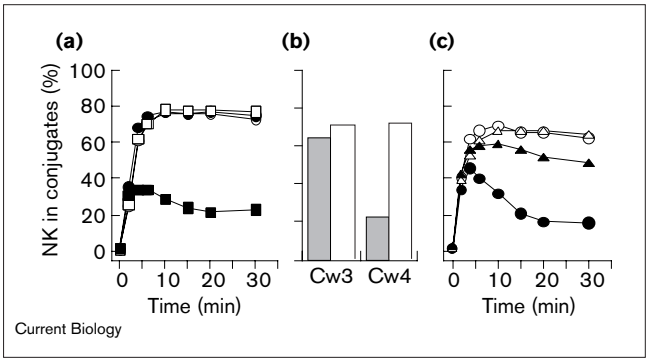
Figure 1



Formation of YTS–target cell conjugates. Typical flow cytometric data for binding of YTS NK cells to 221-Cw3 target cells after **(a)** 0 min and **(b)** 10 min of incubation at 37°C. Green fluorescence on the x axis detects NK cells; red fluorescence on the y axis detects target cells.

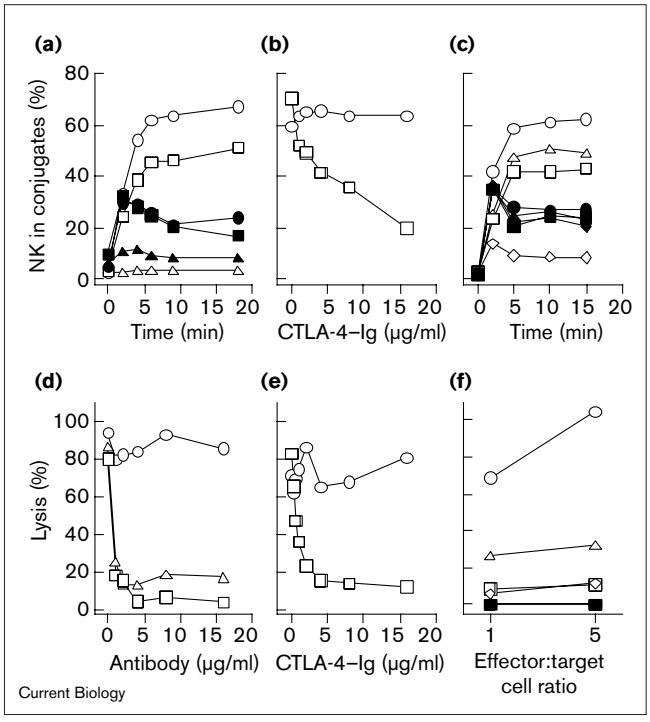
To test whether the tyrosine phosphatase SHP-1 may be involved in inhibition of adhesion, chimeric molecules consisting of KIR2DL1 in which the ITIMs in the cytoplasmic tail had been replaced by full-length SHP-1 were transfected into YTS. This 2DL1–SHP-1 construct blocked conjugate formation with 221-Cw4 cells as effectively as the intact receptor (Figure 2c). A small decrease in conjugate formation was observed with a mutated SHP-1 that has greatly reduced substrate binding and catalytic activity (2DL1–SHP-1(RM), Figure 2c). The two chimeric KIR–SHP-1 molecules had no effect on adhesion to 221-Cw3 cells. These experiments show that SHP-1 is able to inhibit adhesion and, further, that a functional catalytic

Figure 2



(a) Conjugate formation was measured at 37°C for YTS (circles) and YTS-2DL1 (squares) cells with 221-Cw3 (open symbols) and 221-Cw4 cells (filled symbols) cells. **(b)** Conjugate formation of YTS-2DL1 with 221-Cw3 and 221-Cw4 was measured after 10 min at 37°C in the presence of a control IgM MOPC104E (gray bars) or anti-KIR2DL1 IgM antibody HP-3E4 (white bars). **(c)** Conjugates were measured as in (a) using YTS lines expressing the chimeric receptors 2DL1–SHP-1 (circles) or 2DL1–SHP-1(RM) (triangles).

Figure 3



Requirements for adhesion and cytolysis by YTS-2DL1. **(a–c)** Conjugate formation and **(d–f)** target cell lysis by YTS-2DL1 were measured with 221-Cw3 (open symbols) or 221-Cw4 cells (filled symbols). (a,d) The assays were carried out in the presence of MOPC21 (control) IgG (circles), anti-LFA-1 (triangles), or anti-CD28 (squares) antibodies. Antibody concentration in (a) was 10 μg/ml. (b,e) Assays were carried out in the presence of MOPC21 IgG (circles) and CTLA-4–Ig (squares). (c,f) YTS-2DL1 cells were pretreated with PP1 at 0 μM (circles), 2 μM (triangles), 4 μM (squares) and 8 μM (diamonds). PP1 was included in the lysis assay.

domain of SHP-1 is required for this inhibition. In support of this conclusion, a mutant KIR2DL1 with a truncated cytoplasmic tail lacking both ITIMs did not inhibit adhesion to 221-Cw4 cells (D.N.B., M. Peterson, M. Sandusky and E.O.L., unpublished observations. It is still possible that something other than SHP-1 is also recruited by KIR to inhibit adhesion.

The requirements for adhesion of YTS cells to 721.221 cells were evaluated by varying the temperature of the assay and by the addition of blocking antibodies to LFA-1. Adhesion was temperature-dependent, with a noticeable decrease observed at 17°C, and no binding at 4°C (see Supplementary material). Formation of conjugates was blocked by anti-LFA-1 antibodies (Figure 3a), as was the killing of 721.221 cells (Figure 3d). The dependence on temperature and on LFA-1 suggests that adhesion of YTS to target cells uses the 'inside-out' signal through LFA-1 [5]. High-affinity or -avidity LFA-1-mediated binding of T cells to ICAM-1, a ligand of LFA-1, requires an inside-out signal that can be provided by aggregation of the costimulatory molecule CD28 [6], a molecule that is expressed by YTS cells. The cell line YT2C2, another subclone of YT, killed mouse target cells when these mouse cells had been transfected with human B7 (the ligand of CD28) and ICAM-1 [7]. To test whether CD28 contributed to adhesion and killing by YTS cells, we performed antibody blocking experiments. Adhesion was only reduced partially by anti-CD28 antibody (Figure 3a), even though cytolysis was completely abolished (Figure 3d). Therefore, CD28 contributes a critical signal for cytolysis but may be dispensable for LFA-1-mediated adhesion. However, it is also possible that antibody binding to CD28 induced a signal for adhesion but not for cytolysis. To address this possibility we masked B7 on target cells with soluble CTLA-4–Ig fusion protein. Conjugate formation was reduced by 50% at 8 μg/ml CTLA-4–Ig whereas target cell lysis was reduced by half at only 1 μg/ml of CTLA-4–Ig (Figure 3b,e). This result is similar to that of another study in which killing of a B-cell line by YT cells was also reduced by half at 1 μg/ml of CTLA-4–Ig [8]. These results demonstrate the importance of CD28 in activation of cytolysis. They also suggest that CD28 (or the putative NK-specific receptor for B7 [9]) may contribute to adhesion. Although it is clear that lysis of 721.221 cells by YTS is tightly regulated by a signal from CD28, adhesion may be induced by signals coming from other activation receptors.

Given that KIR recruits and activates the tyrosine phosphatase SHP-1 and that a KIR–SHP-1 chimera inhibits adhesion (Figure 2c), it is likely that the target of KIR inhibition includes signaling pathways mediated by tyrosine kinases. A specific inhibitor of Src family kinases, PP1, inhibited both conjugate formation with, and lysis of sensitive target cells (Figure 3c,f). The reduced conjugate

formation with 221-Cw4 cells due to inhibition by KIR was not reduced further by PP1 (Figure 3c). However, the blocking anti-KIR2DL1 monoclonal antibody HP-3E4 eliminated this PP1-resistant adhesion to 221-Cw4 cells (see Supplementary material), indicating that the interaction of KIR2DL1 with HLA-Cw4 on target cells itself contributes some level of adhesion. Our data show that KIR interferes with the ability of NK cells to form conjugates with target cells, despite its own ability to provide some adhesion. Inhibition of adhesion and of cytotoxicity by KIR may be achieved by SHP-1-mediated dephosphorylation of a substrate produced at a proximal step in the activation pathway that lies upstream of signals for adhesion and cytotoxicity. Alternatively, KIR may be able to inhibit signals generated by different receptors, one leading to adhesion and another to target cell killing.

We have established that KIR engagement disrupts conjugation of YTS cells with target cells that bear the appropriate MHC class I molecule. In an earlier study, IL-2-activated human NK clones formed conjugates with target cells to the same extent whether the targets were sensitive or resistant to killing on the basis of MHC class I expression [10]. However, the fraction of NK cells that formed conjugates was about 15%, which is considerably lower than the fraction reported here with the YTS cell line. In agreement with our results, a recent study using live videomicroscopy reported that mouse NK cells spent, on average, less time in conjugates with resistant target cells than with sensitive target cells despite a large variability in the duration of individual conjugates [11]. The concentric distribution of KIR around a core of LFA-1 at the NK–target cell interface [12] may relate to the ability of KIR to disrupt adhesion. Such disruption of NK cell conjugation with resistant target cells may serve the useful purpose of increasing the availabilty of NK cells for the detection of sensitive cells that will elicit a cytotoxic NK response.

An important implication of the new KIR function reported here is that other ITIM-bearing receptors may also serve to regulate cell adhesion, as already proposed [13]. In fact, macrophages from SHP-1-defective *motheaten* mice displayed an increase in adhesion mediated by the αMβ2 integrin [14]. The ITIM-containing receptors SHPS-1 and PIR-B bind SHP-1 and SHP-2 in adherent macrophages and may well provide signals that regulate adhesion [13]. In addition, SHPS-1 associates with Pyk2 [15], a tyrosine kinase that has been implicated in integrin-mediated signaling [16]. These results are consistent with inhibition of adhesion through recruitment of SHP-1 by tyrosine-phosphorylated ITIM-containing receptors such as KIR. ITIMs have been found in many receptors that are expressed in a variety of cell types [1]. All hematopoeitic cells express ITIM-containing receptors, many of which have no known ligand or function [17]. Our results suggest that ITIM-bearing receptors may act as modulators of adhesion in order to regulate important cellular functions such as cell polarization, migration, or detachment from surrounding cells.

Materials and methods

YTS and YTS-2DL1 cells [2] were obtained from G. Cohen (Massachusetts General Hospital, Boston). YTS-2DL1–SHP-1 and YTS-2DL1–SHP-1(RM) cells were produced as described [2]. The arginine at position 459 in SHP-1 was changed to a methionine by site-directed mutagenesis in order to produce mutant SHP-1(RM), which has greatly decreased affinity for substrate and catalytic activity [13]. A KIR2DL1 cDNA was truncated just upstream of the sequence encoding the first ITIM and ligated in frame to the third codon of a SHP-1 cDNA. The resulting 2DL1–SHP-1 chimera contains KIR2DL1 with the first 33 amino acids of its cytoplasmic tail spliced to the SHP-1 molecule with its two SH2 domains. HLA-Cw3, -Cw4, -Cw8, and -Cw15 transfectants of 721.221 were obtained from J. Gumperz and P. Parham (Stanford University). Antibody EB6 (IgG1, reactive with KIR2DL1) was from Immunotech, anti-LFA-1 (IgG1, specific for CD11a) from Upstate Biotechnology, CD28.2 (IgG1, specific for CD28) from PharMingen, MOPC21 (control IgG1) and MOPC104E (control IgM) from Sigma. HP-3E4, an IgM reactive with KIR2DL1 [18], was a gift from M. López-Botet (Hospital de la Princesa, Madrid, Spain). Secondary fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-coupled anti-mouse IgG or IgM were from Jackson Research Laboratories. CTLA4-Ig was from Pharmingen. PP1 was from Biomol Research laboratories.

Conjugate assay

721.221 and YTS cells were labeled with the fluorescent cell linker compounds PKH26-GL (red) and PKH67-GL (green), respectively (Sigma), in 10 μM dye at 5×10^6 cells/ml for 5 min at room temperature. The dye staining reaction was stopped by addition of two volumes of fetal bovine serum. The cells were diluted further in two volumes of medium, washed three times, and rested for at least 1 h at 37°C. Cells were resuspended in cold medium containing 5% FBS. 1×10^5 effector and 2×10^5 target cells were mixed in a final volume of 200 μl in 5 ml round-bottom tubes (Falcon 2052), mixed by gentle vortexing, and centrifuged at 4°C for 3 min at 300 rpm ($25 \times g$). Samples were placed in a 37°C water bath for various times. Cells were resuspended by vortexing, fixed by adding 1 ml of ice-cold 0.5% paraformaldehyde in DPBS, and promptly analyzed on a FACscan (Becton-Dickinson). Results are expressed as percentage of NK cells that formed conjugates with target cells as calculated by the ratio of two-color events to total effector cell events. For antibody inhibition experiments, NK cells were pre-incubated for 15 min at 4°C in twice the final concentration of antibody prior to the addition of target cells. CTLA-4–Ig was incubated with target cells for 15 min at 4°C prior to the addition of NK cells. For inhibition with PP1, NK cells were pre-incubated for 30 min at 37°C in the indicated concentration of inhibitor. Target cell lysis was measured in a standard ^{51}chromium release assay as described [4].

Supplementary material

Supplementary material including data on the YTS-2DL1 cell; on conjugate formation with 221-Cw15 and -Cw8 cells; on temperature dependence of conjugate formation; and on adhesion mediated by the KIR2DL1–HLA-Cw4 interaction, and additional methodological details is available at http://current-biology.com/supmat/supmatin.htm.

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